Induction of Polyclonal Prostate Cancer-Specific CTL Using Dendritic Cells Transfected with Amplified Tumor RNA[1]

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Polyvalent cancer vaccines targeting the entire antigenic spectrum on tumor cells may represent a superior therapeutic strategy for cancer patients than vaccines solely directed against single Ags. In this study, we show that autologous dendritic cells (DC) transfected with RNA amplified from microdissected tumor cells are capable of stimulating CTL against a broad set of unidentified and critical prostate-specific Ags. Although the polyclonal CTL responses generated with amplified tumor RNA-transfected DC encompassed as a subcomponent a response against prostate-specific Ag (PSA) as well as against telomerase reverse transcriptase, the tumor-specific CTL were consistently more effective than PSA or telomerase reverse transcriptase CTL to lyse tumor targets, suggesting the superiority of the polyclonal response. Although tumor RNA-transfected DC stimulated CTL, which recognized not only tumor but also self-Ags expressed by benign prostate tissue, these cross-reactive CTL were exclusively specific for the PSA, indicating an immunodominant role of PSA in the prostate cancer-specific immune response. Our data suggest that tumor RNA-transfected DC may represent a broadly applicable, potentially clinically effective vaccine strategy for prostate cancer patients, which is not limited by tumor tissue availability for Ag preparation and may minimize the risk of clonal tumor escape. *The Journal of Immunology,* **2001, 166: 2953–2960.**

Multiple prostate or prostate cancer-associated Ags have been identified, thus fostering continued efforts to develop clinically effective immunotherapy strategies. Accordingly, it has been shown that autologous dendritic cells (DC)[3] pulsed with peptides specific for prostate-specific Ag (PSA) (1), prostate-specific membrane Ag (2), or telomerase reverse transcriptase (TERT) (3) are capable of stimulating potent CTL in vitro, suggesting their role as potential candidate Ags for prostate cancer immunotherapy. Nonetheless, there is growing recognition that there may be significant advantages to inducing immune responses against a broad spectrum of Ags expressed by the patient's autologous tumor, rather than targeting single, defined tumor Ags (4, 5). Recent research indicates that the emergence of Ag loss mutants frequently arising at metastatic sites may render a substantial proportion of tumors resistant to single Ag-specific immunotherapy (6–8). Alternatively, a polyvalent vaccine directed against multiple Ags may not only lessen the chances for clonal tumor escape but also may represent a more potent approach since CTL against the unique, patient-specific Ags are induced, which apparently represent the dominant rejection Ags in the antitumor response (9). Furthermore, targeting of multiple MHC class I- as well as class II-restricted epitopes expressed by tumor cells may further stabilize or prolong effective T cell responses in the tumor-bearing host by inducing or maintaining T cell memory (10). Although DC pulsed with unfractionated antigenic material in the form of tumor lysates or tumor extracts have shown efficacy in stimulating T cell responses against multiple unidentified tumor Ags (11, 12), this strategy was limited, however, by the requirement of large amounts of tumor tissue for Ag generation. This drawback particularly applies to prostate cancer patients in whom access to tumor tissue is often limited, even in advanced tumor stages. Moreover, considerable tissue heterogeneity may further complicate the procurement of pure tumor tissue which can be used for Ag preparation; thus, most protocols using tumor-derived antigenic materials for vaccination exclude patients carrying small or microscopic tumors. However, these patients may represent the most appropriate group to study vaccine strategies since large tumor burdens have been shown to inhibit antitumor immunity (13).

As a potential solution to this problem, it has been shown that vaccination with tumor RNA-transfected DC can be remarkably effective in stimulating CTL and tumor immunity in in vitro and in vivo models (14). Since Ags encoded by tumor RNA can be amplified from few tumor cells, RNA-transfected DC would allow treatment of cancer patients even with minimal tumor burden without having to identify the Ags involved (15). The objective of this study was to develop a broadly applicable and ultimately clinically effective vaccine strategy that would allow the induction of T cells directed against a broad repertoire of prostate tumor Ags. We show that by combining laser capture microdissection (LCM), PCR, and reverse transcription, nonlimiting amounts of mRNA-encoded tumor Ag(s) can be generated. Autologous DC cultured from cancer patients can be effectively sensitized with the amplified tumor

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[3] Abbreviations used in this paper: DC, dendritic cell; PSA, prostate-specific Ag; TERT, telomerase reverse transcriptase; H&E, hematoxylin and eosin Y; LCM, laser capture microdissection; GFP, green fluorescent protein; NPT, normal prostatic tissue; SSCP, single-stranded conformational polymorphism; TIL, tumor-infiltrating lymphocyte.

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mRNA as demonstrated by their ability to stimulate potent polyclonal T cell responses in vitro. The potency of this approach was further suggested by experiments demonstrating that tumor-specific CTL were consistently more effective then PSA- or TERT-specific CTL to recognize and lyse tumor targets. We further show that the tumor-specific CTL not only recognize endogenously expressed Ags but also Ags expressed by nonmalignant prostatic tissue. Interestingly, these autoreactive T cells were exclusively specific for the self-Ag PSA, but not for other shared Ags, suggesting an immunodominant role of PSA Ags in the prostate-specific antitumor immune response. This study provides a preclinical rationale for further investigation of a potentially clinically effective and broadly applicable treatment for prostate cancer patients, which does not require the characterization of the relevant antigenic profile in each patient and will not be limited by tumor tissue availability for Ag preparation.

Materials and Methods

Human subjects and tissue procurement

PBMC, prostate tumor, and nonmalignant control tissues were collected following informed consent from human subjects treated on protocols approved by the Institutional Review Board at our institution. Prostate-derived tumor material was retrieved by needle biopsy using an automatic 18-gauge biopsy system (Microvasive, Boston, MA) or from surgical materials obtained during radical prostatectomy. The tissues were snap frozen in liquid nitrogen, cryosectioned, and stained with Harris hematoxylin and eosin Y (H&E) to allow histopathological analysis. The tumor content within each biopsy specimen was quantified by one of us (M.S.B.) using light microscopy. Control RNA was extracted from two histologically distinct tissues: 1) microdissected nonmalignant prostatic tissues (NPT) containing prostatic epithelium and interstitial stroma and 2) PBMC.

Laser capture microdissection (LCM)

Cryosectioned and H&E-stained tissue sections were analyzed at ×10 magnification using the laser capture microscope (PixCell; Arcturus Engineering, Mountain View, CA). Cancer tissue was identified microscopically and the LCM transfer cap was placed over the region of interest. For cryosections of 10-μm thickness, the following settings were used: 9-μm spot size, 50-mW pulse power, and 50-ms pulse duration. Dependent on the tumor content within each tissue section, microdissection was conducted in the fashion of positive or negative selection. In positive selection, the identified neoplastic tissue was transferred via the LCM cap to a 0.5-ml microcentrifuge tube containing lysis buffer as the required first step for RNA extraction. In negative selection, stromal and benign epithelial tissues were microdissected and removed while the remaining cancer tissue was directly lysed on the glass slide using a micropipette and then used for RNA extraction. All microdissection procedures were conducted within 2–3 h after cryosectioning and H&E staining.

RNA extraction, amplification, and in vitro transcription

Total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's specifications. To attest to their intactness, all RNA preparations were subjected to electrophoresis in 1% agarose gel under denaturing conditions with clear visualization of intact 18S and 28S ribosomal bands following ethidium bromide staining. Total tumor RNA was reverse transcribed using the SMART method (Clontech, Palo Alto, CA). Briefly, first-strand cDNA synthesis was primed with a modified oligo(dT) primer (5′-AAG-CAG-TGG-TAA-CAA-CGC-AGA-GTA-$CT_{(30)}N_{-1}$N-3′ with N = A,C,G, or T and N_{-1} = A, G, or C) and a strand switch primer (5′-AAG-CAG-TGG-TAA-CAA-CGC-AGA-GTA-CGC-GGG-3′) and reverse transcribed using SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD) for at least 1 h at 42°C. For DNA amplification, the PCR 3′ primer 5′-AAG-CAG-TGG-TAA-CAA-CGC-AGA-GT-3′, a T7 promoter/SMART primer (5′-TAA-TAC-GAC-TCA-CTA-TAG-GGA-GGA-AGC-AGT-GGT-AAC-AAC-GCA-GAG-T-3′), the Advantage cDNA Polymerase Mix (Clontech), and the following cycling parameters were used: 95°C for 1 min, 95°C for 15 s, 65°C for 30 s, 68°C for 6 min for 20 cycles, and 4°C Hold. The quality of the cDNA was evaluated using ethidium bromide-stained 1.2% agarose gel.

In vitro transcription was performed using the T7 mMessage mMachine Large Scale In Vitro Transcription kit (Ambion, Austin, TX). Briefly, transcription mix, ribonucleotide mix, purified cDNA, and T7 enzyme mix were mixed and incubated at 37°C for at least 4 h. The DNA template was degraded by adding DNase I (RNase free) and incubating at 37°C for 15 min. The reaction was stopped by adding RNase-free H_2O and transcription stop mix. The RNA was purified using RNeasy Columns (Qiagen) according the manufacturer's protocol.

DC generation from peripheral blood precursors

For DC culture, we adopted techniques previously described (16, 17) implementing modifications to allow processing of clinical-grade cellular material using defined serum free-media and supplements. Briefly, a concentrated leukocyte fraction was generated through a 2-h restricted peripheral blood leukapheresis processing 6–8 liters of blood with each collection. The leukapheresis product was further separated by density gradient centrifugation over polysucrose/sodium diatrizoate (Histopaque; Sigma, St. Louis, MO), and cells were resuspended in serum-free AIM-V medium (Life Technologies). PBMC were incubated at 2×10^8 cells/30 ml in T-150 culture flasks in a humidified incubator for 2 h at 37°C to allow plastic adherence. The adherent cell fraction was used for DC culture by incubation in serum-free AIM-V medium supplemented with recombinant human IL-4 (500 U/ml) and recombinant human GM-CSF (800 U/ml; R&D Systems, Minneapolis, MN). After 7 days of culture, cells were harvested and phenotypically characterized to assure they met the typical phenotype of immature DC: MHC I^{pos}, MHC II^{pos}, $CD80^{low}$, $CD86^{low}$, $CD83^{neg}$, $CD3^{neg}$, $CD14^{neg}$, $CD16/CD56^{neg}$, $CD19^{neg}$. DC preparations fulfilling these phenotypic criteria were used for subsequent RNA transfection.

RNA transfection of cultured autologous DC

RNA pulsing of autologous DC was performed by simple coincubation of DC in RNA solution without any transfection reagent. In brief, DC were washed twice in PBS, counted, and spun at 300 × *g* for 10 min. They were resuspended in AIM-V medium and incubated in RNA containing solution (3.0 μg RNA per 2×10^6 DC in 2 ml) for 45 min in a humidified incubator at 37°C in 5% CO_2. If subsequently cultured in the absence of T lymphocytes, RNA-loaded DC did not significantly up-regulate DC maturation markers, i.e., CD80, CD86, CD83, CD40, and HLA-DR.

In vitro cytotoxicity assay

The Ag-presenting function of the RNA-pulsed DC was assessed by measuring the induction of primary CTL responses in a standard ^{51}Cr cytotoxicity assay. The T cell-enriched nonadherent fraction of PBMC obtained following the DC plastic adherence step was used for CTL generation. Nonadherent PBMC were cultured in RPMI 1640 cell culture medium supplemented with 20 U/ml human IL-2 and 10 ng/ml human IL-7 (PeproTech, Rocky Hill, NJ). Cells were stimulated twice, 8 days apart, with autologous DC transfected with prostate tumor RNA at a stimulator:effector ratio of 1:10. After 16 days of culture, effector cells were harvested without further separation for microcytotoxicity assays. As determined by flow cytometric analysis, 77 ± 10% of these effector cells were $CD3^{pos}$ and ~40% were of the $CD3^{pos}/CD8^{pos}$ phenotype. Target cells included DC transfected with prostate tumor or benign prostatic tissue derived-RNA, PSA RNA (1), DC pulsed with the PSA-specific, HLA-A2-restricted peptides PSA-1 (aa 141–150: FLTPKKLQCV) and PSA-3 (aa 154–163: VISNDVCAQV) (kindly provided by Jeffrey Schlom, National Cancer Institute, Bethesda, MD), DC transfected with RNA encoding the jelly fish green fluorescent protein (GFP) (18), or the human prostate cancer cell line LnCAP (American Type Culture Collection, Manassas, VA). No attempts were made to increase MHC class I expression on these LnCAP target cells by IFN-γ treatment.

Target cells were labeled with sodium chromate-51 in saline solution ($Na^{51}CrO_4$; NEN Life Science Products, Boston, MA) by incubation of 2×10^6 transfected DC or LnCAP cells in 1 ml RPMI 1640 with 100 μCi of ^{51}Cr for 1 h at 37°C in 5%CO_2. After three washes, 5×10^3 ^{51}Cr-labeled targets and serial dilutions of effector cells at various E:T ratios were incubated in 200 μl of RPMI 1640 in 96-well U-bottom plates. These plates were incubated for 6 h at 37°C in 5%CO_2. Then, 50 μl of the supernatant was harvested and released ^{51}Cr was measured with a scintillation counter. Cold target inhibition assays were performed at an E:T of 40:1 and unlabeled targets were added as specified. Spontaneous release was <15% of the total release by detergent in all assays. SD of the means of triplicate wells was <5%.

Single-stranded conformational polymorphism (SSCP) analysis of T cell receptors

TCR β-chain SSCP analysis was performed according to the method of Andrews et al. (19). Briefly, lymphocyte total RNA was reverse transcribed

using random hexamers and Moloney murine leukemia virus-reverse transcriptase. The resulting cDNA was then used as template for multiplexed PCR using paired V-β family-specific primers and a HEX-labeled β-chain constant region primer according to the method of Yassai and Naumova (20), but with 40 cycles of amplification. PCR products were then denatured in formamide and resolved by nondenaturing gel electrophoresis. Gels were then analyzed using an automated FMBioII fluorescence scanner (Hitachi, Brisbane, CA).

Results

Total tumor RNA-transfected DC stimulate tumor-specific CTL

Monocyte-derived DC were generated from peripheral blood precursors using a clinical protocol described elsewhere (16, 17). Immature DC expressing the phenotype MHC I^{pos}, MHC II^{pos}, $CD80^{low}$, $CD86^{low}$, $CD83^{neg}$, $CD3^{neg}$, $CD14^{neg}$, $CD16/CD56^{neg}$, $CD19^{neg}$ were used for RNA transfection, conducted by short-term coincubation of RNA with cultured DC without using any transfection agent. The ability to transfect DC with "naked" RNA reflects their immature phenotype since treatment of DC with TNF-α or CD40 ligand before transfection down-regulates their stimulatory capacity (21). We first determined whether cultured DC transfected with total prostate tumor RNA are capable of stimulating CTL responses against prostate cancer-associated Ags in vitro.

Tumor samples were obtained by needle biopsy from an HLA-$A2^+$ patient with a large prostate mass from which total RNA was extracted. For this and all of the succeeding experiments, the tumor content within each biopsy specimen was quantified by an experienced uropathologist based on light microscopic analysis of each sample following cryosectioning and H&E staining. In this experiment, histological analysis revealed all samples to consist of tumor cells only. For CTL priming, autologous PBMC were stimulated twice with autologous DC transfected with tumor RNA 8 days apart. The ability of tumor-specific CTL to recognize tumor Ags was analyzed using standard cytotoxicity assays. In this and previous studies, RNA-transfected DC were not only used as stimulators but also served as specific or control targets in cytotoxicity assays. To validate their use as surrogate tumor targets, HLA-A2-matched human LNCaP prostate carcinoma cells were used as additional targets in cytotoxicity assays.

As shown in Fig. 1*A*, total prostate tumor RNA-transfected DC were capable of stimulating robust T cell responses recognizing and lysing the total tumor RNA-transfected DC, whereas DC targets transfected with the irrelevant RNA species (PBMC RNA or GFP RNA) were not lysed. Prostate tumor-specific CTL were comparable to PSA-specific CTL stimulated with PSA RNA-transfected DC in recognizing and lysing PSA-expressing targets (Fig. 1, *A* and *B*), indicating that the levels of PSA RNA in the total tumor RNA pool were sufficient to stimulate PSA-specific CTL. Importantly, the tumor-specific CTL were superior in recognizing and lysing tumor targets to CTL stimulated with PSA RNA-transfected DC, suggesting that tumor-specific CTL represent a polyclonal response providing more effective antitumor activity than T cell responses directed against a single Ag in the form of PSA.

The experiment shown in Fig. 1*C* demonstrates that the tumor-specific CTL are not only capable of recognizing and lysing tumor RNA-transfected DC targets but also human LNCaP cells (HLA-$A2^+$, PSA^+), albeit less efficiently, suggesting the presence of shared Ags between the autologous tumor and the HLA-matched cell line. Similarly, CTL stimulated with PSA RNA-transfected DC were able of lysing human LNCaP cells and PSA RNA-transfected DC targets with similar efficacy, suggesting the equivalence of the DC targets to tumor targets (Fig. 1*D*). Similar observations were made in other studies in which PSA (1), TERT (22), or tumor-specific CTL (23) were capable of lysing tumor cells as well as Ag-presenting DC with similar efficacy.

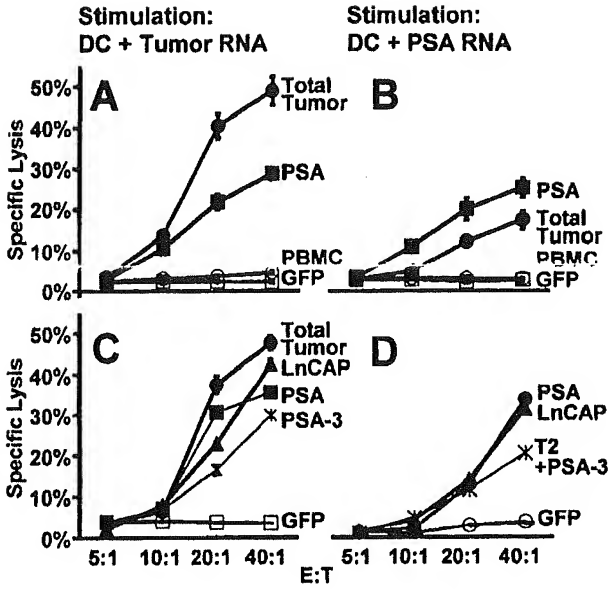

FIGURE 1. Autologous DC transfected with autologous tumor RNA stimulate CTL, which recognize tumor- and prostate-specific Ags. Autologous PBMC were stimulated twice with DC transfected with total cellular RNA derived from autologous prostate tumor (*A* and *C*) and with PSA RNA (*B* and *D*). PBMC were assayed for the presence of CTL recognizing and lysing the following DC transfected with autologous total prostate tumor RNA (total tumor) or DC loaded with PSA RNA (PSA) or PSA-3 peptide (PSA-3). Additionally, T2 cells pulsed with PSA-3 peptide (T2 + PSA-3) and LnCAP cells were used as targets. As controls DC transfected with PBMC RNA (PBMC) or GFP RNA (GFP) were used.

In summary, these experiments not only demonstrate that CTL priming in vitro against prostate tumor Ags can be successfully accomplished by using prostate tumor RNA-transfected DC, they also validate their use as surrogate targets in CTL assays. This is of particular importance since primary prostate tumor cells are notoriously difficult to culture and expand ex vivo, rendering their use for immunological monitoring of prostate cancer patients impractical (24).

Enrichment of tumor-derived mRNA improves CTL stimulation

Unfortunately, most prostate tumors are small and frequently interspersed with benign interstitial stromal cells potentially necessitating further tumor cell enrichment to yield pure tumor material for RNA extraction. Therefore, we investigated whether the presence of normal tissue within a tumor sample source would impact on the efficacy of tumor RNA-transfected DC to stimulate tumor-specific CTL in vitro. To generate RNA preparations with decreasing tumor RNA content, we performed serial dilutions of pure (100% tumor content) prostate tumor RNA (patient B) with autologous PBMC RNA. PBMC RNA was chosen as an irrelevant RNA species since DC transfected with PBMC RNA reproducibly fail to induce CTL against their cognate targets as well as tumor targets in vitro (manuscript in preparation). Cultured autologous DC were then transfected with either pure or sequentially diluted tumor RNA and subsequently used to stimulate tumor-specific CTL in vitro. As specific or control targets, DC transfected with pure prostate tumor RNA, PBMC RNA, or GFP RNA were used. As shown in Fig. 2*A*, a tumor RNA concentration-dependent CTL response against tumor targets was observed. DC transfected with 100% pure prostate tumor RNA and DC transfected with 60% prostate

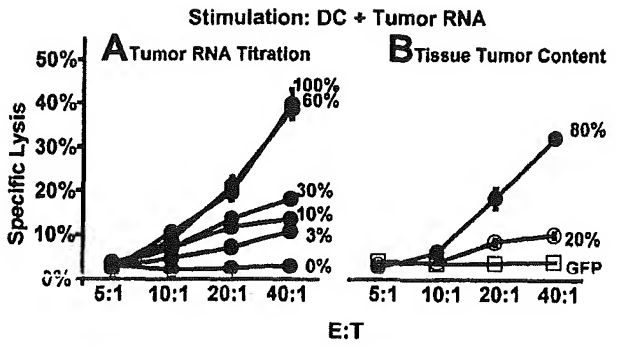

FIGURE 2. Enrichment of tumor-derived mRNA improves CTL stimulation. DC were transfected with serial dilutions. Pure prostate tumor RNA with PBMC RNA (range, 0–100%) derived from the same patients (*A*) were used for CTL priming. The CTL were analyzed for their ability to recognize and lyse DC transfected with prostate tumor RNA (100% tumor content). A similar experimental setup was used in the experiment shown in *B*, in which DC transfected with RNA extracted from tumor biopsies containing 80 or 20% tumor content were used for CTL stimulation. DC transfected with 80% tumor RNA served as targets.

tumor RNA-stimulated CTL that were equally effective to recognize and lyse DC targets transfected with pure tumor RNA. However, DC loaded with a lower prostate tumor RNA content (30, 10, and 3%) had a decreasingly lower stimulatory capacity.

Using a clinically more relevant setting, we used two needle biopsy-derived prostate tissue cores with either 80 or 20% tumor content (patient C) as RNA source. DC transfected with RNA extracted from these samples were used to stimulate CTL in vitro. As specific or control targets, DC transfected with 80% prostate tumor RNA and GFP RNA were used. As shown in Fig. 2*B*, DC transfected with 80% tumor RNA were capable of eliciting a robust CTL response to recognize their cognate targets (DC transfected with 80% tumor RNA), whereas DC transfected with 20% tumor RNA failed to stimulate CTL to recognize and lyse these tumor targets. The results from these initial experiments show that tumor RNA-transfected DC are effective stimulators of tumor-specific CTL in vitro. They also underscore the importance that RNA-encoded tumor Ags of sufficient purity are required to stimulate these tumor-specific T cells, emphasizing the need for further tumor enrichment in tumor samples with limited tumor content.

Generation of amplified, in vitro-transcribed mRNA from microdissected prostate tumor tissue

From the experiments reported thus far, it becomes apparent that the amounts of total prostate tumor RNA, which can be extracted from small tumor samples or needle biopsies, are insufficient to transfect large numbers of DC required for clinical trials. However, it has been shown that tumor RNA can be amplified and used to transfect DC, which then become capable of stimulating tumor-specific CTL in vitro (15). Therefore, we developed a sequential clinically applicable protocol which allows the selective procurement of prostatic tumor cells from microscopic tumor samples followed by RNA extraction, PCR-based amplification, and in vitro transcription. Our primary goal was to generate nonlimiting amounts of tumor mRNA, which faithfully encodes for the entire antigenic spectrum expressed by prostate tumor cells. We used LCM (25) conducted in the fashion of either positive or negative selection to selectively isolate small cancer nests from H&E-stained frozen tissue sections (Fig. 3, *A–C*) or from needle biopsy derived-tissue cores (Fig. 3, *D* and *E*). Using cryosections of 10-μm thickness, we were able to routinely extract 1.5 μg of total

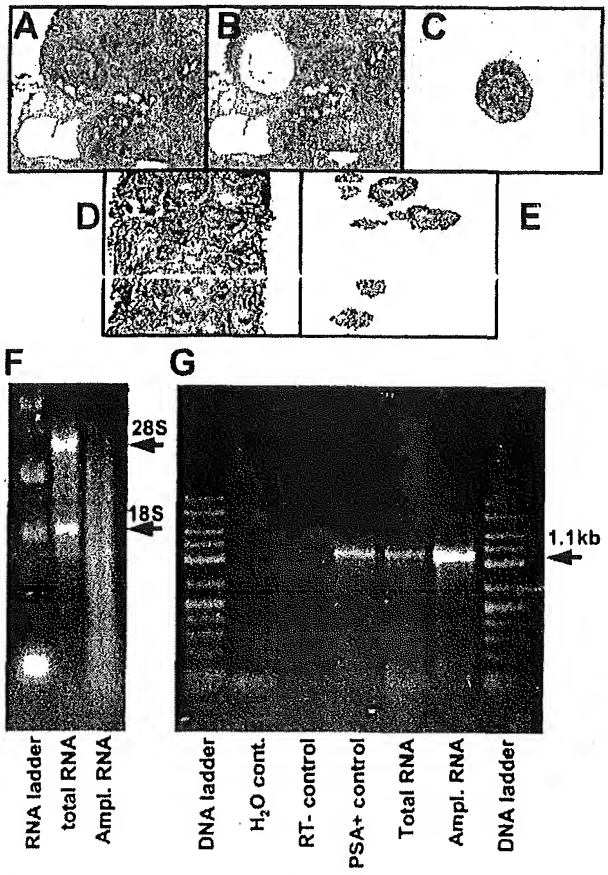

FIGURE 3. Generation of amplified, in vitro-transcribed mRNA from microdissected prostate tumor tissue. Examples of LCM transfer from surgically obtained tumor tissue section (*A–C*) or from prostatic needle biopsy-derived material (*D* and *E*). Cryosections were dehydrated, mounted on a glass slide, and stained with H&E (*A* and *D*). A transparent thermoplastic film (ethylene vinyl acetate polymer) is applied to the surface of the tissue section on a standard glass histopathology slide. A carbon dioxide laser pulse then specifically activates the film above the cells of interest. Focal adhesion allows selective procurement of the targeted cells (*C* and *E*). Using a transfer cap, target cells can be selectively transferred and placed into a microcentrifuge tube for further processing. RNA was extracted from microdissected tumor cells and subjected to agarose gel electrophoresis. Although the predominant RNA species present before amplification (total RNA) correspond to the two ribosomal RNA species (18S, 28S), amplified mRNA migrates as a heterogeneous population representing the polyadenylated mRNA species within prostate tumor cells (*F*). To further attest to the intactness of the amplified tumor mRNA, RT-PCR for PSA was performed with successful amplification of a distinct 1.1-kb band (*G*).

RNA/mm^2 microdissected prostate tumor tissue. Dependent on specimen size or the tumor content within each sample, up to 10 slides were required to yield ~0.50 μg of total tumor RNA. The amplified tumor cDNA was then converted into RNA using in vitro transcription systems as described previously (15). As expected, agarose gel electrophoresis demonstrated that the predominant RNA species present before amplification correspond to the two ribosomal RNA species, while the amplified RNA migrates as a heterogeneous smear corresponding in size to the entire mRNA population including the high molecular mass RNA species present in prostate cancer cells (Fig. 3*F*). To demonstrate that the RNA-coding sequences were preserved at full-length during the amplification/transcription procedure, amplified mRNA was successfully used as template for PSA-specific RT-PCR as evidenced by the appearance of a 1.1-kb gene product (Fig. 3*G*).

DC transfected with amplified tumor RNA efficiently stimulate polyclonal, tumor-specific cytotoxic T cell responses

We next determined whether the amplified and in vitro-transcribed tumor mRNA is capable of sensitizing DC to stimulate CTL in a manner comparable to total tumor RNA isolated directly from the tumor cells. Specifically, we were concerned that the thermal energy produced by the laser pulse during microdissection may result in damage to the isolated cells and their genetic material, potentially impacting on RNA yield and quality. To address these questions, three DC preparations (patient B) were generated consisting of DC transfected with 1) pure total prostate tumor RNA (Fig. 4*A*), 2) amplified tumor RNA derived from nonmicrodissected tissue (Fig. 4*B*), or 3) amplified tumor RNA derived from microdissected tissue (Fig. 4*C*) and used for CTL induction. As shown in Fig. 4, *A* and *B*, DC transfected with amplified prostate tumor RNA were equally as effective as DC transfected with total tumor RNA to stimulate CTL which recognized and lysed total tumor RNA or amplified tumor RNA-transfected DC, but not GFP RNA-transfected DC targets. Clearly, microdissection did not negatively impact on our ability to generate and amplify functionally active tumor mRNA, since DC transfected with amplified tumor RNA derived from nonmicrodissected (Fig. 4*B*) or microdissected (Fig. 4*C*) tumor RNA were equally effective in stimulating potent tumor-specific CTL in vitro to recognize and lyse their cognate targets. This demonstrates that faithful amplification and transcription of the mRNA content from microdissected tumor tissue can be achieved and that DC can be effectively sensitized with the amplified tumor RNA to stimulate tumor-specific CTL in vitro. We further show that DC loaded with amplified, microdissected tumor RNA were not only capable of stimulating CTL to recognize and lyse tumor targets but also DC targets transfected with RNA-encoding PSA or TERT, respectively (Fig. 4*C*). This suggests that DC transfected with amplified, microdissected tumor RNA stimulate T cell responses directed against multiple tumor-associated Ags including PSA or TERT.

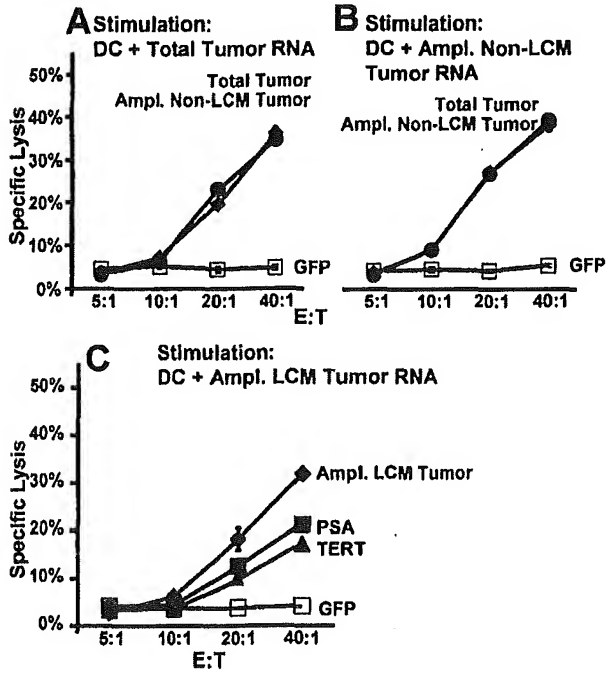

FIGURE 4. Autologous DC transfected with amplified RNA from microdissected prostate tumor tissue stimulate CTL that recognize autologous tumor, PSA, and TERT. PBMC were stimulated twice with DC transfected with three different RNA preparations and tested for the presence of tumor-specific CTL: 1) total tumor RNA derived from nonmicrodissected prostate tumor tissue (*A*), 2) RNA amplified from nonmicrodissected prostate tumor tissue (ampl. non-LCM tumor RNA, *B*), and 3) RNA amplified from microdissected prostate tumor tissue (ampl. LCM tumor RNA, *C*). These DC preparations not only served as stimulators for CTL priming but also served as targets in cytotoxicity assays. Additional targets included DC transfected with PSA RNA, TERT RNA, or GFP RNA.

Further evidence that tumor RNA-transfected DC can stimulate polyclonal T cell responses was provided from cold target inhibition experiments in which tumor-specific CTL were generated from PBMC stimulated with DC transfected with amplified, microdissected tumor RNA. These DC were used as ^{51}Cr-labeled (hot) targets, which were competed out against unlabeled (cold) targets consisting of 1) GFP RNA-transfected DC, b) DC pulsed with PSA peptides (PSA-1 and PSA-3), 3) PSA RNA-transfected DC, 4) TERT RNA-transfected DC, 5) human LNCaP cells, and, finally, 6) amplified tumor RNA-transfected DC.

As shown in Fig. 5*A*, DC transfected with amplified tumor RNA completely blocked the induced, tumor-specific CTL from lysing their tumor targets (DC transfected with amplified tumor RNA) while GFP RNA-transfected DC failed to inhibit lysis. DC pulsed with the HLA-A2-restricted PSA peptides PSA-1 and PSA-3 and PSA RNA-transfected DC (encoding for multiple PSA epitopes) were only partially effective in competing out the CTL response against amplified tumor RNA-transfected DC targets. TERT RNA-transfected DC were also, albeit less effective then PSA RNA-transfected DC, able to inhibit the CTL response stimulated by amplified tumor RNA-transfected DC (Fig. 5*B*). The tentative conclusion from this experiment is that only a fraction of the CTL generated by tumor RNA-transfected DC are directed against PSA or TERT.

To further characterize the effector cells involved in the tumor-specific T cell response, the T cell clonality was evaluated by PCR amplification of the rearranged TCR β-chain (TCRβ) using Vβ-specific primers. The SSCP of the PCR products leads to differential migration rates through nondenaturing gels (26). Thus, each discrete band corresponds to a clonally expanded T lymphocyte that utilizes a specific β-chain as part of its TCR. DC transfected with 1) amplified prostate tumor RNA, 2) PSA RNA, or 3) HLA-A2-restricted PSA-3 peptide were generated and used to stimulate CTL from PBMC. CTL were first analyzed in a cytotoxicity assay and found to be functionally active by recognizing and lysing tumor and PSA Ag-expressing DC targets (data not shown). RNA extracted from these CTL was further analyzed for their individual rearrangement of the TCR Vβ chain using a total of 24 PCR primers specific for each Vβ family followed by SSCP analysis. Unstimulated PBMC or prostate tumor-infiltrating lymphocytes (TIL) were included in this analysis. As shown in Fig. 6, we detected highly constant TCR SSCP-banding patterns for Vβ25, which were found in CTL stimulated by DC loaded with PSA-3 peptide, PSA RNA, and tumor RNA but not in the naive PBMC (Fig. 6, marker 2). This suggests that PSA epitope-specific rearrangements are not only preserved in CTL stimulated by PSA RNA-transfected DC but are also present in tumor-specific CTL. Interestingly, these PSA epitope-associated TCR SSCP patterns were also found in TIL, suggesting the presence of PSA-specific CTL precursors within tumor tissues. Consistent with the cold target experiments described above, the SSCP patterns of T cells stimulated by DC transfected with prostate tumor RNA was more diverse when compared with PSA-specific and unstimulated PBMC spectratype patterns as demonstrated for Vβ2 (Fig. 6, marker 4). This suggests that tumor RNA-transfected DC may stimulate broader sets of T cell specificities, including those directed against PSA.

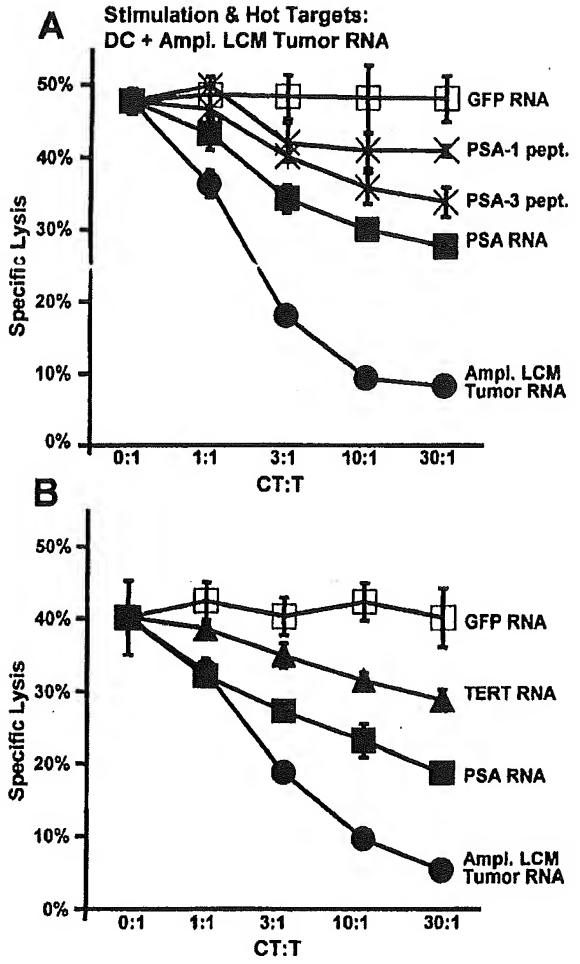

FIGURE 5. Autologous DC transfected with amplified RNA from microdissected prostate tumor tissue stimulate a polyclonal tumor-specific CTL response. Tumor-specific CTL were generated from PBMC using DC transfected with RNA amplified from microdissected prostate tumor as stimulators. In both experimental settings, these DC were used as radioactively labeled (hot) targets which were competed out against the following unlabeled (cold) targets: 1) GFP RNA-transfected DC, 2) DC pulsed with PSA peptides (PSA-1 and PSA-3), 3) PSA RNA-transfected DC, 4) TERT RNA-transfected DC, 5) human LNCaP cells, and 6) DC transfected with RNA amplified from microdissected prostate tumor.

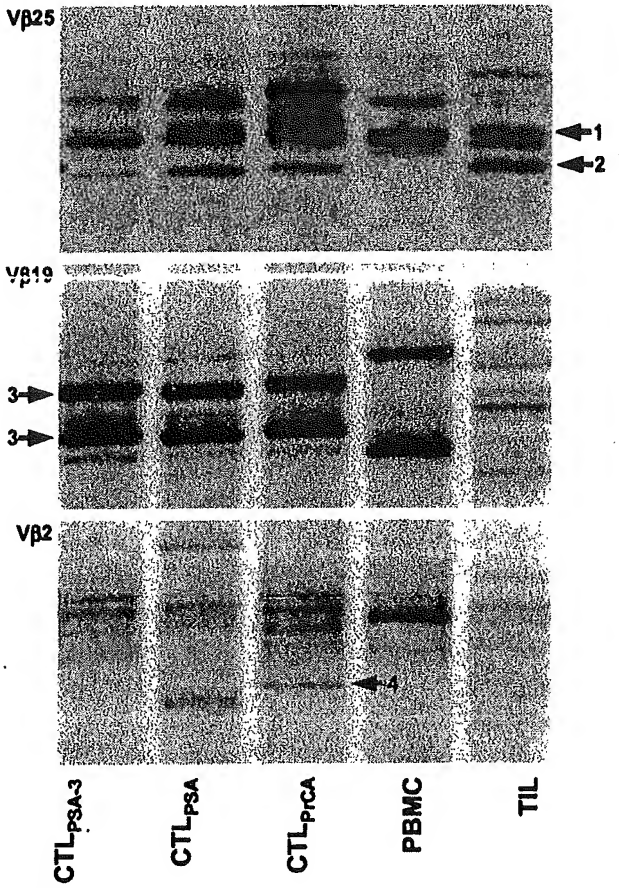

FIGURE 6. SSCP analysis of TCR-β chain. PBMC from a prostate cancer patient were stimulated twice with autologous DC transfected with RNA from autologous prostate tumor tissue (CTL_{PrCA}), PSA RNA (CTL_{PSA}), or autologous DC pulsed with the HLA-A2-restricted PSA-3 peptide (CTL_{PSA-3}). PCR using Vβ-specific primers was performed followed by SSCP analysis using total RNA extracted from above DC preparations as a template. RNA from unstimulated PBMC or prostate tumor-specific TIL were used as controls. As exemplified on Vβ25, Vβ19, and Vβ2, the following SSCP banding patterns were identified (see arrows): 1) patterns present in all preparations; 2) patterns present in PSA-specific T cells (stimulated by PSA RNA and PSA peptide-pulsed DC) and TIL; 3) present in tumor-specific CTL, PSA-specific CTL, but not in TIL or PBMC; and 4) present in tumor-specific CTL but not in any other preparation.

Induction of CTL DC transfected with prostate tumor RNA stimulate CTL cross-reactive with Ags expressed by nonmalignant prostate tissue

We next sought to determine whether DC transfected with amplified tumor RNA stimulate T cell responses, which are tumor specific and solely recognize prostate tumor Ags, or whether these CTL can also cross-react with shared Ags expressed by nonmalignant prostatic tissue. For this analysis, two DC preparations were generated by transfecting cultured DC (patient D) with autologous amplified RNA derived from 1) microdissected prostate cancer tissue or 2) microdissected NPT, which included benign epithelial cells and surrounding interstitial stroma. Both DC preparations were used for CTL priming in vitro and as targets in cytotoxicity assays. DC transfected with PSA RNA, TERT RNA, PBMC RNA, and GFP RNA represented additional targets in these cytotoxicity experiments.

DC transfected with microdissected, amplified prostate tumor RNA reproducibly stimulated potent CTL responses against tumor, PSA, and TERT Ag-expressing DC targets. Interestingly, these CTL also recognized and lysed DC transfected with amplified RNA derived from NPT but not GFP or PBMC-transfected DC (Fig. 7*A*).

As shown in Fig. 7*B*, we also attempted to induce CTL specific for NPT by transfecting autologous DC with amplified microdissected NPT RNA. Although the lytic levels obtained with these NPT RNA-derived CTL were less pronounced than with tumor RNA-transfected DC, a consistent recognition of tumor RNA, PSA RNA, and NPT RNA-transfected DC targets was observed, while expectedly TERT (not overexpressed in normal prostate tissue), PBMC, and GFP Ag expressing DC targets were not lysed. These experiments indicate that DC transfected with amplified prostate tumor RNA stimulate CTL which not only recognize tumor Ags but also Ags expressed by NPT, providing indirect evidence that in fact shared Ags may exist among normal and cancerous prostate tissues.

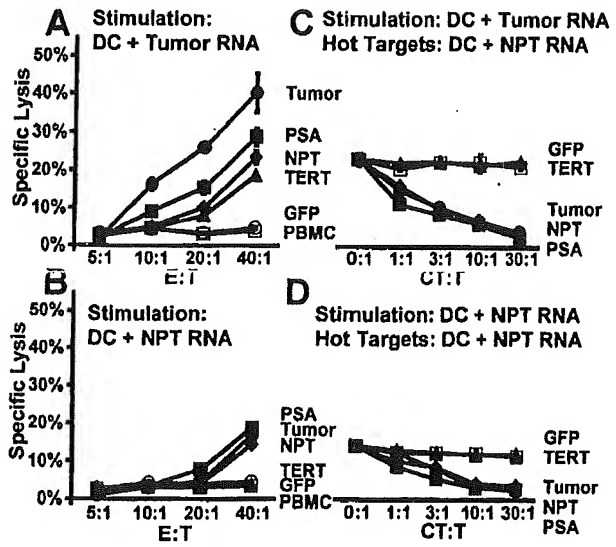

FIGURE 7. The CTL responses recognizing normal prostate Ags are directed against PSA Ags. CTL were stimulated from autologous PBMC by coculture with the following DC preparations: DC transfected with RNA amplified from microdissected prostate tumor tissue (tumor, *A* and *C*) and RNA amplified from NPT (NPT RNA, *B* and *D*). CTL were assayed in cytotoxicity assays using the above DC preparations or DC transfected with PSA RNA, TERT RNA, PBMC RNA, or GFP RNA as targets (*A* and *B*). These CTL were further analyzed in cold target inhibition experiments (*C* and *D*) using DC transfected with RNA amplified from microdissected NPT as radioactively labeled targets. These hot targets were competed out against the following unlabeled (cold) targets: GFP RNA-transfected DC, PSA RNA-transfected DC, TERT RNA-transfected DC, and DC transfected with RNA amplified from microdissected prostate tumor (tumor) or NPT.

The CTL responses recognizing normal prostate Ags are exclusively directed against PSA

To define the nature of these cross-reactive CTL, we performed cold target inhibition experiments using CTL effectors stimulated by either tumor RNA-transfected DC (Fig. 7*C*) or by DC transfected with NPT RNA (Fig. 7*D*). In both experimental settings, DC transfected with NPT RNA served as labeled (hot) targets, which were competed out against unlabeled (cold) targets consisting of GFP RNA-transfected DC, TERT RNA-transfected DC, amplified PE RNA-transfected DC, amplified tumor RNA-transfected DC, and PSA RNA-transfected DC. Regardless whether tumor-specific or NPT-specific CTL were used as effectors cells, GFP RNA- and TERT RNA-transfected DC had no impact to compete out the NPT-specific DC from lysing NPT RNA-transfected DC, whereas DC transfected with PSA RNA, amplified tumor RNA, and NPT RNA completely inhibited this response.

These experiments provide evidence that T cells specific for PSA are responsible for the cross-reactivities seen between tumor and normal prostate Ags. These T cells can recognize PSA Ags present on both normal and malignant prostate tissue, suggesting that PSA may represent an immunodominant Ag involved in the polyclonal prostate or prostate cancer-specific immune response.

Discussion

The overall objective of this study was to develop an efficient and clinically applicable protocol to generate CTL against multiple prostate tumor Ags using DC transfected with tumor RNA. The main advantage of using tumor RNA-encoded Ags is that only minute amounts of tumor tissue are required for DC transfection since RNA can be amplified from few cancer cells using molecular biology techniques such as PCR and in vitro transcription. Here, we show that LCM is a powerful tool to selectively procure pure tumor cells from microscopic tumor samples from which tumor RNA was extracted and amplified. Besides these technological advantages, we further demonstrated that DC transfected with amplified tumor RNA are remarkably effective in stimulating potent T cell responses against a broad repertoire of Ags, which apparently included CTL subsets specific for PSA and TERT. Importantly, we show that tumor RNA-transfected DC were superior than PSA or TERT RNA transfected DC to induce T cell reactivities against tumor targets, suggesting that a polyclonal antitumor response directed against multiple tumor-derived Ags would be more potent then a monoclonal or oligoclonal response directed against a single Ag.

These findings may have several implications for the design and the application of human cancer vaccines: 1) The fact that tumor RNA can be faithfully amplified even from microscopic tumor samples broadens the spectrum of vaccination to patients with limited tissue availability or to tumor systems in which potent tumor Ags have not yet been identified. 2) In addition to its broad applicability, this technology may allow prolonged vaccination cycles or continuous boosting, strategies which are thought to be critical to maintain potent antitumor immunity in cancer patients (10). 3) Polyvalent tumor vaccines in the form of tumor RNA-transfected DC may not only represent a more potent approach but also may preempt the formation of tumor escape mutants through down-regulation or complete loss of individual tumor Ags. 4) It is conceivable that that DC transfected with RNA-encoded unfractionated tumor Ags may not only stimulate potent CTL responses, but also tumor-specific $CD4^+$ T cells which play an important role in the induction and persistence of MHC class I-restricted antitumor CTL.

One concern of immunizing with unfractionated, tumor-derived antigenic mixtures as compared with strategies using defined Ags represents the potential induction of autoimmunity by breaking tolerance to self-Ags expressed by tumor cells (27). Although in our study tumor RNA-transfected DC elicited CTL were capable of recognizing and lysing both tumor as well as benign prostate tissue targets, these cross-reactive T cell responses were exclusively directed against the shared self-Ag PSA. Assuming that other Ags besides PSA exist which are shared between prostate cancer and benign prostatic cells (known as differentiation Ags in melanoma studies), our data indicate that these Ags are either insufficiently expressed by nonmalignant prostate cells or may contain only subdominant T cell epitopes in the presence of (dominant) PSA Ags. These findings are consistent with previous studies in which renal tumor RNA-transfected DC failed to stimulated CTL, which recognized normal tissue Ags including Ags expressed by benign renal parenchyma and conversely DC transfected with RNA from nonmalignant tissues failed to stimulate CTL against cancer and nonmalignant targets (manuscript in preparation). Since prostates are nonessential for life, total autoimmunity against prostate tissue would be perfectly acceptable if these immune responses would remain restricted to prostate-specific Ags but not to Ags associated with normal housekeeping products. Whether autoimmunity with pathological consequences will ultimately limit the use of RNA-transfected DC vaccines in cancer patients must further await careful analysis in clinical trials.

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